1.0 OBJECTIVE

The purpose of the development test with the sea urchin, *Strongylocentrotus purpuratus*, is to determine if sea water, pore water, sea surface microlayer, or other samples cause abnormal development of exposed embryos relative to embryos exposed to control or reference samples (US EPA 1995). The test may also be used to determine the concentration of a test substance that causes abnormal development. Test results are reported as treatment (or concentration) that produce statistically significant abnormal development or as a concentration of test substance that causes 50 percent abnormal development (EC50).

2.0 EQUIPMENT

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate SOP.

2.1 Urchin Collection and Culture

• Tanks, trays, or aquaria for holding adult sea urchins, e.g. standard salt water aquarium (capable of maintaining sea water at 15° C), with appropriate filtration and aeration system.

• Air pump, airlines, and air stones -- for aerating water containing adult urchins (for static systems and emergency aeration for flow-through systems).

2.2 Test Initiation

• Wash bottles

• Environmental chamber (15 ± 1°C, ambient laboratory illumination for 16 hours/day)

• Pipettes, automatic

• Hemacytometer

• Sedgwicke-Rafter counting cell

• Mixing Plunger (for mixing gametes)

• Graduated cylinders

• 0.5 M KCl

• Syringe and 24 gauge needle for injecting urchins

2.3 Test Termination

• Inverted or Compound microscope

• Data sheets (one set per test) -- for data recording.

- Formaldehyde, 37% (Concentrated Formalin)
- Fume hood -- to protect the analyst from effluent or formaldehyde fume
- Counter, two unit, 0-999 -- for recording counts of embryos and larvae.

2.4 Water Quality

- Meters and probes for measuring pH, dissolved oxygen, and ammonia
- Refractometer for measuring salinity
- Thermometers (glass mercury thermometer and continuously recording chart thermometer)
- Centrifuge, spectrophotometer and reagents for measuring sulfide (see sulfide SOP)
- Graduated pipettes (10 ml) and hand pipette pump for water quality sampling
- Gloves and appropriate safety gear (see MPSL lab safety manual)

2.5 Dilution Water

Dilution water consists of ambient Granite Canyon seawater, filtered to 1 µm, at ambient salinity (33-34‰). This water is used to prepare eggs and sperm for toxicity tests, and for diluting test solutions.

3.0 EXPERIMENTAL DESIGN

Aquatic toxicity tests can be used as screening tools or as part of more comprehensive studies to assess water quality. Careful consideration must be given to site characteristics, reference site selection, field replication, choice of synoptic measures, seasonal factors, and comprehensive planning and peer review to determine that study designs are adequate to meet program objectives.

This laboratory toxicity test consists of five replicate scintillation vials for each sample. Samples are occasionally diluted into several concentrations. Vials are arranged randomly, and each receives 250 urchin embryos. The quality of test embryos and testing conditions is determined through concurrent testing of reference toxicants (positive controls), seawater control, and brine control (negative controls). Testing of reference sites is recommended to demonstrate the suitability of test porewaters in the absence of toxic contaminant concentrations. Test conditions of temperature and photoperiod are controlled as indicated below, and pH, H_2S, NH_3, salinity, dissolved oxygen, and temperature are measured at the beginning and end of the exposure.

4.0 SAMPLE PREPARATION

Label scintillation vials as indicated on the randomization sheet generated for the test. Label another set of vials, three each with the sample number of each sample, to be used for water quality measurements at the end of the test. Determine the salinity of the test solutions. Be sure to stir all samples before

measuring salinity, and measure salinity immediately after stirring. Samples salinities below 32‰ are adjusted to 34‰ using hypersaline brine made from frozen seawater or artificial salts. Check the pH of brine. If necessary, adjust the brine pH by adding acid or sodium hydroxide until it is between 7.5-8.5.

Using the random number sheet, aliquot 10 ml of sample to scintillation vials and water quality containers. Minimize sample exposure to sunlight (never leave samples in direct sunlight), and schedule loading times to avoid prolonged sample exposure to temperatures above 15°C.

5.0 CONTROLS

5.1 Seawater and Brine Controls (Negative Controls)

A seawater control consisting of 1-µm filtered Granite Canyon water should accompany each batch of samples. If any salinity adjustments were made a brine controls must also be prepared. The brine control must contain the same amount of brine as the lowest salinity sample.

5.2 Reference Toxicant Tests (Positive Controls)

Conduct a concurrent reference toxicant test each time an urchin development test is conducted (standard procedure). The reference toxicant test similar exposure time, and provides data on the relative sensitivity of each batch of urchin embryos.

Prepare a stock solution of 10,000 µg Cu per liter by carefully weighing 0.0268 grams of copper chloride ($CuCl_2$), and carefully pouring the weighed solid into one liter of distilled water in a plastic volumetric flask. Cap tightly and mix thoroughly. Pipette the following volumes of stock solution into a clean volumetric flask (dilution flask) and add water (ambient salinity, 15°C) to make one liter of test solution at the following concentrations, respectively: 0.0 ml for controls, 0.32 ml for 3.2 µg/L, 0.56 ml for 5.6 µg/L, 1.0 ml for 10 µg/L, 1.80 ml fo 18 µg/l, and 3.20 ml for 32 µg/L (refer to the laboratory copper dilution sheet for *S. purpuratus*). Place the reference toxicant test containers in the constant temperature room, cover, and allow to equilibrate.

6.0 TEST INITIATION

6.1 Test Organisms

Newly fertilized embryos of the sea urchin, *Strongylocentrotus purpuratus* are used in the development toxicity test. Animals may be collected in the field or obtained from a commercial supplier. *S. purpuratus* is distinguished from *S. franciscanus*, a less common cogener, by it's purple to occasionally green color. Urchins can be maintained easily in aquaria or other tanks provided with running seawater. Urchins will eat a wide variety of marine algae, but prefer giant brown kelp, *Macrocystis pyrifera*. To ensure year-

round spawning, broodstock are held at ambient seawater temperature, in complete darkness. Water quality parameters should be monitored weekly and salinity maintained at 32 ± 2‰. It is preferable to separate urchin broodstock by sex to prevent accidental spawning. The sex of the urchins may be determined at the time of spawning. After animals are spawned for toxicity tests, males and females should be placed in separate culture tanks. Once these animals are re-conditioned they may be spawned for use in later toxicity tests.

6.2 Test System

Five replicates are used for all treatments. Each replicate contains 10 mL of test solution. A brine control is necessary if any salinity adjustments are required. The brine control should contain a volume of brine equal to the brine volume used to adjust the sample with the lowest salinity. Make the brine control by first adding enough fresh Arrowhead Spring Water to adjust Granite Canyon dilution water to a salinity equal to that of the lowest salinity sample. Then adjust this sample back up to 34 ± 2‰ using an appropriate volume of brine, calculated on the Salinity Adjustment Worksheet.

6.3 Collection and Preparation of Gametes

To perform the test repeatedly, quality gametes must first be collected, and then diluted to the appropriate density of embryos for addition to the test vials.

6.3.1 Spawning of Urchins

• Place ten urchins on several layers of paper towels on a clean surface.
• Inject each urchin with 0.5 cc of 0.5 M KCl in the soft tissue surrounding the Aristotle's lantern and gently shake the animals once or twice to stimulate gamete release.
• Re-inject with 0.5 cc of 0.5 M KCl, any urchins that have not spawned after 10 minutes.
• Females release orange-colored eggs, and males release cream-colored sperm.

6.3.2 Gamete Quality

• Eggs must be inspected for uniformity and roundness. Select only females with uniformly round eggs that lack follicles. Do not use a batch of eggs if a high proportion of germinal vesicles are present or if eggs are released from females in large clumps.
• Eggs and sperm should be washed off the urchins with a squirt bottle filled with 1-µm filtered seawater. Gametes should be rinsed into separate beakers. Use special care to avoid cross contamination between sperm and eggs.
• Place one drop of eggs onto a well slide and add a small amount of sperm to test fertilization. Check for a fertilization membrane. If no fertilization membrane is present, isolate new eggs.

• Place the eggs in the constant temperature room (15° C) until ready for counting.

6.3.6 Fertilization of Gametes

Use the urchin spawning worksheet to determine the appropriate sperm and egg dilution factors. Sperm and eggs are combined based on worksheet equations. Embryos are added at a density of 250 (+/-10%) per vial.

7.0 TEST TERMINATION

To terminate the test, use a toxic dispenser or disposable pipette, add 1.0 mL 37 % buffered formalin to each sample to give a final formalin concentration of 4%. Gently shake containers to mix. As an alternate fixative, 0.5 mL of 1.0 % gluteraldehyde may be added to each test container. Test containers may now be capped and stored for later evaluation.

8.0 DATA COLLECTION AND TABULATION

Count a minimum of 100 individuals (e.g., embryos, plutei, etc.) per sample using hand counter with multiple keys (such as a blood cell counter). Use one key to indicate normal larvae and another to indicate abnormal larvae. Due to the shape of the larvae, their orientation may need to be adjusted in order to determine normality.

8.1 Normal Larvae

Normally developed larvae have the following characteristics:

• Four spicules that extend at least half the distance from the base to the apex.

• A gut which demonstrates signs of differentiating into three chambers. In some cases it is not possible to distinguish all three chambers of the gut. If the apex of the gut appears lobed and constricts distally, then normal gut development may be inferred.

8.2 Abnormal Larvae

Abnormal or inhibited larvae may fit into any one of the following categories:

• Pathological prehatched: unfertilized eggs and fertilized eggs with the membrane still visible.

• Pathological hatched: larvae that have no fertilization membrane and demonstrate an extensive degree of malformation or necrosis. Most larvae appear as dark balls of cells or disassociated blobs.

• Retarded: larvae at blastula and gastrula stages that have no gut differentiation or have underdeveloped spicules.

• Gut abnormalities: larvae that are otherwise normal with exogastrulated guts, lacking differentiation or lacking guts.

• Skeletal abnormalities: misshapen larvae as a result of missing spicules or underdeveloped spicules, rods growing in abnormal directions or extraneous spicules.
Note: Spicules that are slightly separated at the apex might be caused by formalization and should not be considered abnormal.
Calculate Percent Normal Development for each control replicate test. Test acceptability is 70% normal development in the reference test seawater control.

9.0 DATA HANDLING AND TEST ACCEPTABILITY

Immediately after test termination, check the data sheet to determine whether dilution water and salinity controls have acceptable normal development (mean of 80% or greater). If not, notify the project officer without delay.

Tests with temperature, salinity, or dissolved oxygen measurements outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Acceptable temperatures are 15 ± 1°C; acceptable dissolved oxygen concentration is 60-100% saturation.

10.0 REFERENCES

U.S. Environmental Protection Agency. 1995. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. Office of Research and Development. EPA/600/R-95/136. August 1995

11.0 TEST SUMMARY

Species:	*Strongylocentrotus purpuratus*
Test Duration:	72-96 hours
Endpoint:	Normal Development
Renewals:	None
Organism Source:	Wild-caught adult broodstock that has been maintained in the laboratory in constant darkness and free flowing seawater
Test Salinity:	Ambient ± 2‰
Test Temperature:	15°± 1°C
Dilution water:	1 µm filtered seawater at 15°C.
Lighting:	Constant illumination.
Replication:	5 replicates per sample, plus a three for water quality
Test Containers:	20 ml glass scintillation vials

***Strongylocentrotus purpuratus* 96-Hour Larval Development Test**

Loading:	Approximately 250 embryos per vial
Water Quality:	pH, D.O. temp°, salinity ‰, NH_3, H_2S.
Reference Toxicant:	Copper Chloride ($CuCl_2$)
Stock Solution:	0.0268 g in 1 liter of distilled water (= 10,000 µg/L).
Dilutions:	0, 3.2, 5.6, 10, 18, and 32 µg/L
Safety:	Wear protective clothing; read applicable MSDS, be familiar with the lab safety manual prior to testing.
Quality Control:	Fill out all data sheets completely. Be familiar with QA Project Plan prior to testing.
Acceptability Criteria:	Controls: ≥ 70% Salinity range: Ambient ± 2‰ Temperature range: 14° to 16°C. Dissolved oxygen range: 4.91 to 8.19 mg/L.